

## CHEMOPREVENTIVE ACTIVITY OF THE ETHANOLIC EXTRACT OF *ASTRAEUS HYGROMETRICUS* (PERS.) MORG. ON EHRLICH'S ASCITES CARCINOMA CELLS

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Mechanisms on the chemopreventive activity of the ethanolic extract against Ehrlich's ascites carcinoma cells were delineated. Determination of cell cycle phase distribution by flowcytometry indicated an enhancement in the number of cells in the sub-G0/G1 population suggesting cancer cell apoptosis. These findings were additionally confirmed by nuclear staining that exhibited distinctive morphological features of apoptosis. Our data also revealed an increase in the expression of pro-apoptotic protein p53 in the carcinoma cells. Pro-apoptotic gene Bax was up-regulated during p53-mediated apoptosis. Down-regulation of the anti-apoptotic protein Bcl-2 was observed ensuing in decrease of the Bcl-2/Bax ratio. All these effects might be bestowed on the presence of high amount of flavonoids and phenolic compounds.

(Received November 22, 2011; Accepted February 6, 2012)

*Keywords:* Apoptosis, *Astraeus hygrometricus*, Bax, Bcl2, Cancer, Ehrlich's ascites carcinoma, Mushroom, p53

### 1. Introduction

Cell cycle checkpoints assist to lead the systematic progression and completion of events such as chromosome segregation, DNA replication and initiation of differentiation vital in eukaryotic cells [1]. The first event that occurs when the fate of the cells is determined to either proliferation or differentiation is the arrest of the cell cycle. In the G0/G1 phase of the cell cycle, the decision of cells to differentiate is made [2]. The Bcl-2 family members are also vital regulators of apoptosis. These oncoproteins are classified in two groups: anti-apoptotic that restrains apoptosis and pro-apoptotic that encourages it. The members form heterodimers to inactivate each other. The up-regulation of Bax and down-regulation of Bcl-2 expression at the protein level have been observed during apoptosis. Fascinatingly, Bcl-2 over expression renders cells defiant to apoptosis when it homodimerizes, whereas, Bcl-2/Bax heterodimer development stimulates apoptosis. These combined associations among members of the Bcl-2 family are important in ascertaining the inclination of a cell to go through apoptosis [3]. Thus, cell cycle arrest and the initiation of apoptosis in the carcinoma cells turn out to be the foremost indicators of apoptogenic effects. Most notably, the majority of bioactive substances from natural products have been reported to exercise their chemopreventive activity against cancer by triggering tumor cell apoptosis and blocking cell cycle progression. Large arrays of phenolic substances, mainly those present in dietary and medicinal plants, have been reported to possess noteworthy anticarcinogenic and chemopreventive effects. The greater part of these naturally occurring phenolics hold anti-inflammatory and anti-oxidative properties that are responsible for their chemopreventive activity [4-6].

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There has been a recent upsurge of interest in mushrooms as a source of biological active compounds of medicinal value, including antioxidant, anti-cancer, anti-inflammatory, cardioprotective and hepatoprotective agents [7-13]. These newly found compounds, termed 'mushroom nutraceuticals', are extractable from either the fungal mycelium or fruiting body and represent an important component of the expanding industry of mushroom biotechnology. *Astraeus hygrometricus*, a wild ectomycorrhizal mushroom, consumed by the local people with the belief that this mushroom can prevent age related disorders. Recent scientific investigations on this mushroom reveal that a water soluble glucan has strong splenocyte activation properties. Ethanolic extract shows potent *in vitro* free-radical scavenging activity [14], and *in vivo* anti-inflammatory [10], hepatoprotective [13] and cardioprotective activities [15].

Here, an attempt has been made to evaluate the chemopreventive effect of the ethanolic extract (Ee) of *A. hygrometricus* on Ehrlich's ascites carcinoma (EAC) cells grown in the peritoneal cavity of Swiss albino mice.

## **2. Experimental**

### **2.1. Sample collection and preparation**

Basidiocarp of *A. hygrometricus* was collected from the local market and from the sal (*Shorea robusta* G.f.) forests of Bankura and West Midnapore, West Bengal, India, cleaned and washed thoroughly and extracted according to Biswas *et al.*, 2010 [10]. Fresh mushrooms were randomly selected into three samples of 150 g each and air-dried in an oven at 40°C for 48 h. Dried powdered mushroom sample was extracted by stirring with 200 ml of ethanol at 30°C for 24 h at 150 rpm and filtering through Whatman No. 4 filter paper. The residue was then extracted twice with another 200 ml of ethanol as described above. The total extract was then rotary evaporated to dryness at 40°C and redissolved in ethanol to a concentration of 10 mg/ml and stored at -20°C for further use.

### **2.2. Phytochemical analysis**

Total phenolic content in the extract was measured according to the method of Slinkard [16] using Folin-Ciocalteu reagent and keeping pyrocatechol as the standard. The total phenolic concentration was expressed as mg pyrocatechol equivalents (PE)/100 g fresh weight. Flavonoid concentration of the extract was also determined using quercetin as standard [17]. Total flavonoid concentration was expressed as mg quercetin equivalents (QE)/100 g fresh weight.

### **2.3. Animals**

Healthy male Swiss albino mice of approximately the same age weighing about 20 g were used for the study. They were fed with standard diet and water *ad libitum*. The animals were maintained according to the guidelines recommended by Animal Welfare Board and approved by our Institutional Animal Ethical Committee. All procedures complied with the Declaration of Helsinki, as revised in 1996.

### **2.4. Acute toxicity studies**

The standard conditions of the mice were maintained during the experiment. They were housed in polypropylene cages maintained under standard conditions (12 h light/dark cycle; 25°±3°C temperature, 35-60% relative humidity). The extract was fed orally with increasing dose up to 3000 mg/kg body weight.

### **2.5. Tumor models**

Swiss albino mice (~20 g each; 10 mice in each group) were randomly divided into different groups including: (i) normal set (non-tumor-bearing); (ii) tumor-bearing set which were intra-peritoneally injected with  $1 \times 10^5$  exponentially grown EAC; (iii) extract-treated (150 mg/kg body weight) non-tumor-bearing set, and (iv) extract-treated (150 mg/kg body weight) tumor-bearing set. Untreated mice received drinking water instead of the extract. The treatment was started 30 days prior to EAC injection to evaluate the chemopreventive activity of the extract. On 21<sup>st</sup> day after EAC cells administration, the animals were sacrificed [18]. To determine the extract concentration to be used in the *in vitro* assay, prior to the experiment, the cell counts of the EAC cells were taken into consideration after treatment with ascending doses from 50 to 200 mg/kg body weight.

### 2.6. Isolation of EAC from mice peritoneal cavity

The EAC cells were isolated from the peritoneal cavity of tumor-bearing mice (control or treated). Three milliliters of sterile phosphate buffered saline (PBS) was injected into the peritoneal cavity of the mice and the peritoneal fluid containing the tumor cells was withdrawn, collected in sterile Petri dishes and incubated at 37°C for 2 h to separate non-adherent cells. The non-adherent population was aspirated out gently. Viable EACs from each mouse were counted in haemocytometer by trypan blue exclusion test. The viable EAC cells were processed for further experiments [19, 20].

### 2.7. Detection of apoptosis by flowcytometry

EAC cells were permeabilized and nuclear DNA was labeled with propidium iodide (PI, 125 µg/ml) and utilized for the analysis of cell cycle phase distribution under FACS (BDFACS Calibur), fluorescence activated cell sorter equipped with 488 nm Argon laser light source and 623 nm band pass filter (linear scale). Ten thousand events were acquired and analyzed. A histogram of DNA content (x-axis, PI-fluorescence) versus counts (y-axis) has been displayed [20].

### 2.8. Phenotypic analysis of EAC cells by confocal microscopy

EAC cells were fixed and nuclear DNA was stained with DAPI, 4', 6'-diamidino-2-phenylindole (0.2 µg/ml for 15 min at room temperature). A laser scanning confocal microscope (Zeiss LSM 510 META) was used to visualize apoptotic cells [21].

### 2.9. Flowcytometric analysis of expression of pro- and anti-apoptotic proteins

EAC cells from mice were fixed and permeabilized as described earlier and used further for the determination of the expression of pro-apoptotic proteins p53 and Bax or anti-apoptotic protein Bcl-2. Cells ( $1 \times 10^5$ ) from each group were incubated either with polyclonal anti-p53 or anti-Bcl-2 or anti-Bax (1 µg/ml) primary antibody for 1 h at room temperature. They were then treated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Cells were washed thoroughly with PBS and analyzed on FACS equipped with 488 nm Argon laser light source and a 515 nm band pass filter for FITC-fluorescence. Acquired ten thousand events were analyzed and histogram plot of FITC-fluorescence (x-axis) versus counts (y-axis) has been shown in logarithmic fluorescence intensity.

### 2.10. Western blotting

EAC lysate was loaded into a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was transferred to nitrocellulose membrane and blocked with non-fat milk in PBS containing Tween-20 prior to p53, Bax and Bcl-2 primary antibody treatments. The protein of interest was visualized by treating with alkaline phosphatase (AP) conjugated secondary antibody. Equal loading of protein in each lane was established by  $\alpha$ -actin antibody probing.

## 3. Results

### 3.1. Phytochemical analysis

The extract was found to have high concentration of phenolic compounds and flavonoids at concentrations of  $80 \pm 7.9$  mg PE/100 g fresh weight and  $40 \pm 9$  mg QE/100 g fresh weight, respectively.

### 3.2. Acute toxicity studies

The *A. hygrometricus* ethanolic extract when administered up to a dose of 3000 mg/kg body weight did not exhibit any symptoms or signs of either toxicity or mortality.

### 3.3. Effect of the extract on tumor cell number

We assessed the effect of the extract on the number of EACs in the extract untreated or treated tumor bearing mice. Administration of the extract decreased the tumor cell number over the untreated negative control set. It was ascertained that the extract lowered the tumor load substantially in a dose dependent manner showing the optimal activity at the dose 150 mg/kg body weight (Table 1). On day 21, a total of  $450 \times 10^6$  EACs were measured in the peritoneal fluid of untreated mice, whereas in 150 mg/kg body weight extract treated group only  $55 \times 10^6$  EACs were found. Hence, further studies were carried out using this dose.

Table 1. Effect of *A. hygrometricus* ethanolic extract on EAC cell number in the peritoneal cavity of tumor bearing mice [Values are mean  $\pm$  SD from 10 mice].

Doses of ethanolic extract (mg/kg body weight)	EAC number ( $\times 10^6$ )		
	1 <sup>st</sup> week	2 <sup>nd</sup> week	3 <sup>rd</sup> week
0	111 $\pm$ 13	300 $\pm$ 24	450 $\pm$ 44
50	98 $\pm$ 18	142 $\pm$ 15	178 $\pm$ 21
100	85 $\pm$ 17	95 $\pm$ 7	102 $\pm$ 12
150	67 $\pm$ 14	63 $\pm$ 5	55 $\pm$ 6
200	66 $\pm$ 5	62 $\pm$ 4	56 $\pm$ 7

### 3.4. Flowcytometric analysis of EAC cell cycle phase distribution

The FACS data described the effect of the extract on cell cycle phase distribution of EAC DNA. On day 21 after EAC inoculation, the content of hypoploid DNA in untreated control set was 5.08% (Figure 1A) which increased to 10.24% in the extract treated set (Figure 1B). Simultaneously, DNA content in G0/G1 phases (41.8% versus 40.77%, Figure 1A versus B) as well as in S and G2/M (37.2% versus 33.7%, Figure 1A versus B) phases decreased in the extract treated set. This outcome suggested that the breakdown of EAC DNA was induced by the extract ensuing tumor killing. The arrest of EAC growth was the apparent ramification.

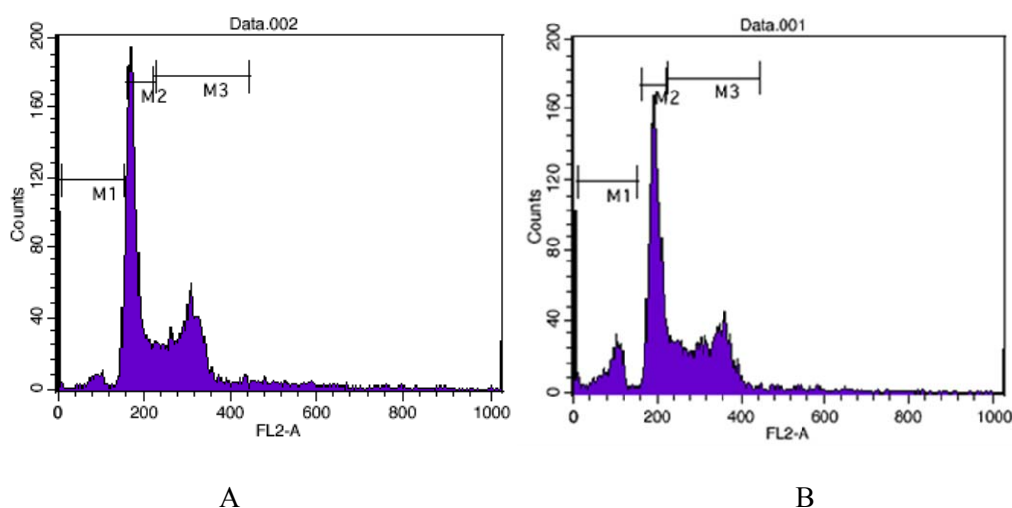


Fig. 1. Flow-cytometric analysis of Ee-induced apoptosis of EAC nuclear DNA was determined by PI staining. Cell cycle phase distribution of nuclear DNA was determined flow cytometrically. Untreated (Control) (A) or Ee-treated (B) EAC nuclear DNA was labeled with propidium iodide. Histogram display of DNA content (x-axis; PI-fluorescence) versus counts (y-axis) has been shown.

### 3.5. Extract induces tumor killing by apoptosis

Comparative confocal microscopic observation was made of DAPI stained EAC cells (Figures 2A and B). Apoptosis characterized phenotypically by chromatin compaction, cell shrinkage, plasma membrane blebbing, fragmentation of DNA and collapse of the cell into small intact fragments (apoptotic bodies) were strongly observed in ethanolic extract treated set (Figure 2B).

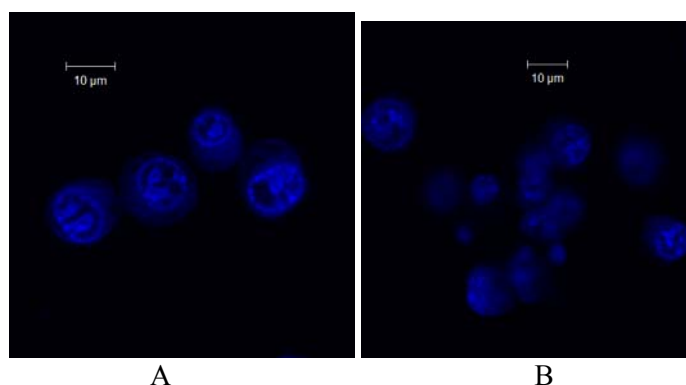


Fig. 2. Oligonucleosomal fragmentation, cell shrinkage, collapse of the cell into apoptotic bodies and nuclear blebbing in the extract-treated EAC is evident by DAPI staining (A, untreated EACs; and B, extract-treated EACs).

### 3.6. Effect of the extract on the expression of pro- and anti-apoptotic proteins

Further, an attempt had been made to uncover the mechanism behind the apoptosis of EAC. It is well established that various pro- and anti-apoptotic proteins play a crucial role in programmed cell death. We examined whether or not the extract has any effect on the expression of pro-apoptotic or growth-arresting proteins, p53 and Bax as well as anti-apoptotic protein, Bcl-2, in our mice model. After the extract treatment, we observed using flowcytometry that the levels of p53 and Bax expression augmented notably in EAC (Figures 3A and B). Furthermore, the level of Bcl-2 decreased (Figure 3C), which lead to the decrease in Bcl-2/Bax ratio. Flowcytometric data was further confirmed by western blot analysis (Figure 3D). It supported the concept that the balance between positive and negative regulators of apoptosis was shifted towards cell death or apoptosis [19] because of the treatment with the extract.

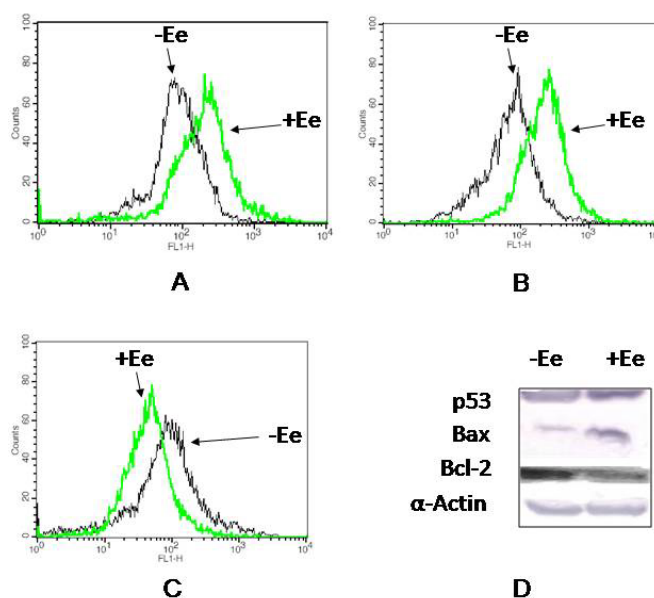


Fig. 3. Effect of the extract on the expression of p53, Bax and Bcl-2 in EAC. EAC cells from the extract-treated and untreated tumor-bearing mice groups were fixed and divided into three parts. (A) First part of the cells from each group was incubated with anti-p53 primary antibody. (B) Second part of the cells was incubated with anti-Bax primary antibody. (C) Third part of the cells was incubated with anti-Bcl-2 primary antibody. All the cells were then incubated with FITC-conjugated secondary antibody. Cells were then analyzed on a flowcytometer and histogram display of FITC-fluorescence (x-axis) versus counts (y-axis) has been shown in logarithmic fluorescence intensity. (D) EAC lysates (untreated or extract-treated) were subjected to western blot analysis with either anti-p53, anti-Bax or anti-Bcl-2 primary antibodies and visualized by AP-conjugated secondary antibody. The  $\alpha$ -Actin band represented equal amount of protein loading.

#### 4. Discussion

The continuing magnitude of the cancer problem and the failure of conventional chemotherapy of the advanced invasive disease to effect the major reduction in the mortality rates indicate that new approaches to the control of cancer are critically needed. The problem lies in our thought. Therefore, we need to consider that cancer is ultimately the end stage of a chronic disease process characterized by abnormal cell and tissue differentiation. This process, which eventually leads to the outcome of invasive and metastatic cancer, is carcinogenesis. We need to focus more on prevention of the disease rather than attempting to cure the end stage of a disease [22].

In recent years, natural antioxidant molecules present in human diet have gained considerable attention as cancer chemopreventive and chemotherapeutic agents. [23-27]. Epidemiological data indicate that vegetables and fruits containing chemopreventive agents could have protective effect against cancer. Furthermore, compared with conventional forms of treatment, the main benefit of using chemopreventive agents is the absence of systemic toxicity and being more appealing. Historically, mushrooms have been shown to possess profound health promoting benefits and recent studies are now confirming their wide spread medical efficacy as well [7-15]. Because of the burning desire to choose the best chemotherapeutic drugs for clinical trial, the recognition of the mechanism(s) of action of a particular drug has assumed high priority.

The hunt for a safe agent that increases the levels of expression of tumor suppressor proteins is a useful and somewhat under-explored approach for cancer chemoprevention. It is currently well known that apoptosis is a type of cell death characterized by active suicide of cells. Our studies have revealed that the extract was successful in imparting growth inhibition, cell cycle de-regulation and apoptosis in EAC cells. It is currently well known that whether a cell becomes committed to apoptosis depends to some extent on the balance among proteins that mediate growth arrest and cell death, e.g., p53, Bax and proteins that uphold cell viability, e.g., Bcl-2 [28, 29]. Within these proteins, p53 has been found to aid apoptosis in diverse cell types. In our study, the extract augmented the p53 expression. The other set of gene products downstream to p53, e.g., Bax, induces apoptosis [30]. Bax, being a member of Bcl-2 family, not only promotes apoptosis, it moreover counters the protecting effect of Bcl-2 [31]. As a matter of fact, over-expression of Bax, an effect that is associated with the development of Bax/Bax homodimers, has been shown to speed up the cell death of murine FL5.12 cells following interleukin-3 withdrawal [32, 33]. In our system, the extract treatment diminished the expression level of Bcl-2 and raised Bax concentration, and thus decreased the Bcl-2/ Bax ratio in these cells. Besides, it was apparent from a variety of studies, that up-regulation of cell growth-modulating genes, upon p53 initiation, might block the cell cycle although enhanced expression of pro-apoptotic factors can override the growth-arresting message and thus leading to apoptosis eventually. In numerous tumor cell lines, p53 has been found to bring on reduced expression of Bcl-2 during the peak of apoptosis [34]. In HNSCC, p53 expression considerably enhanced the expression of Bax, and consequently, growth-arrest and apoptosis took place in these cells [35]. It has moreover been revealed that cells, which are functionally deficient in p53 or show, raised levels of Bcl-2, are comparatively resistive to chemotherapy-induced apoptosis [36]. Here, we had demonstrated that the extract induced apoptogenic signal in a p53-dependent pathway in which over-expression of Bax resulted in tumor cell apoptosis.

The apoptogenic activity of the extract might be due to the presence of flavonoid and phenolic compounds, which had been shown by the preliminary phytochemical analysis of the extract. Furthermore, it had been previously reported that the activation of nitric oxide synthase (NOS) had an inhibitory effect on various types of cancer including EAC [37, 38]. The same ethanolic extract of *A. hygrometricus* was a potent activator of the NOS enzyme as reported earlier by our group [14]. This might be also an added value on the inhibition of EAC.

In conclusion, the present study is the first report showing the effect of ethanolic extract of *A. hygrometricus* in the prevention of EAC in mice model. In summary, based on the present findings, it is tempting to suggest that the ethanolic extract of *A. hygrometricus* and its associated antioxidant and NOS activators may possess a strong potential for its development as a chemopreventive and possibly therapeutic agent against EAC.

#### Acknowledgment

The financial support of the Council of Scientific and Industrial Research (CSIR), New Delhi, is gratefully acknowledged.

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